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Combining Liquid Chromatography with Ozone-Induced Dissociation for the Separation and Identification of **Phosphatidylcholine** Double Bond Isomers

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Abstract

Revealing the inherent molecular diversity of lipid biology requires advanced analytical technologies. Distinguishing phospholipids that differ in the position(s) of carbon-carbon double bonds within their acyl chains presents a particular challenge because of their similar chromatographic and mass spectral behaviours. Here - **for the first time** - we combine reversed-phase liquid chromatography for separation of isomeric phospholipids with on-line mass spectral analysis by ozone-induced dissociation (OzID) for unambiguous double bond position assignment. The customised tandem linear ion-trap mass spectrometer used in our study is capable of acquiring OzID scans on a chromatographic timescale. Resolving the contributions of isomeric lipids, that are indistinguishable based on conventional mass spectral analysis, is achieved using the combination of liquid chromatography and OzID. Application of this method to the analysis of simple (egg yolk) and more complex (sheep brain) extracts reveals significant populations of the phosphatidylcholine PC 16:0_18:1(*n*-7) alongside the expected PC 16:0_18:1(*n*-9) isomer.

Keywords

mass spectrometry, liquid chromatography, phospholipids, double bond isomers, ozone-induced dissociation, collision-induced dissociation

Abbreviations

CID	Collision-induced dissociation
ESI	Electrospray ionization
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
<i>m/z</i>	Mass-to-charge ratio
OzID	Ozone-induced dissociation
PC	Phosphatidylcholine
TIC	Total ion chromatogram
XIC	Extracted ion chromatogram

Author Contributions

TWM, JLC and SJB conceived the project. RLK conducted all experiments and performed the data analysis. JLC provided essential input into the experimental configuration including hardware and software aspects. RLK and SJB wrote the main manuscript text and prepared figures. All authors critically reviewed the manuscript and electronic supplementary materials.

Introduction

At the cellular level, phospholipids are involved in numerous functions including; signalling, modulation of non-lipid biomolecules and maintaining the function and integrity of membranes, among other roles [1-4]. Phospholipids consist of a hydrophilic region (containing a phosphate-bound head group such as choline) connected to a hydrophobic region (containing a glycerol backbone to which two acyl chains are attached). Specific alterations in degree, and location(s) of unsaturation within the fatty acyl chains of phospholipids can alter their cellular functions [5-7]. The potential significance of variation in phospholipid double bond motifs necessitates the development of analytical tools capable of separating and identifying such variants in complex biological extracts.

Contemporary strategies for the separation and identification of phospholipids are focussed on the use of liquid chromatography (LC) generally coupled with mass spectrometry (MS). The use of hyphenated LC-MS techniques for phospholipid analysis has been reviewed and can be broadly classified by the application of either normal- or reversed-phase chromatography [8,9]. Normal phase and hydrophilic interaction LC approaches exploit differences in polar regions of lipid structure and have thus been successfully deployed for the temporal segregation of phospholipids into different headgroup classes. This approach can aid in the quantification of lipids and can be useful in separating lipids of different classes that might share a common elemental composition (*e.g.*, phosphatidylcholines can be separated from isomeric phosphatidylethanolamines). Silver ion chromatography combines the affinity of silver ions for carbon-carbon double bonds with normal phase LC separation conditions. This approach can bring about separations based on differences in the degree, position and stereochemistry of unsaturation in lipids but is most generally successful for low-polarity lipids and has not been widely deployed in phospholipid analysis [10-12]. Reversed-phase LC uses non-polar stationary phases and can bring about separation of a wide variety of lipids, including phospholipids, based on structural differences in their hydrophobic acyl chains. Phospholipids possessing different acyl chain lengths and/or different numbers of double bonds, *e.g.*, phospholipid compositions of PC 36:1 and PC 36:2 can be routinely separated using reversed-phase techniques. Examples of separation of isomeric phospholipids that contain the same number of double bonds that are differentially distributed across the acyl chains, *e.g.*, PC 18:1_18:1 and PC 18:0_18:2, are also relatively common [13-16]. In contrast, analytical separations of phospholipid double bond positional isomers – where the acyl chains are themselves isomeric – by reversed-phase LC have proven more challenging. Prepared mixtures of two synthetic phosphatidylcholine double bond positional isomers, PC 18:1(*n*-9)/18:1(*n*-9) and PC 18:1(*n*-12)/18:1(*n*-12), have been successfully separated by reversed-phase LC using a C₈ column and, more recently, using charged surface hybrid C₁₈ columns [17,18]. While these analytical demonstrations are important, very few

studies have differentiated between endogenous double bond positional isomers in complex biological extracts. In one such example, Brouwers and co-workers extracted lipids from the parasitic worm, *S. Mansoni*, and subjected these extracts to two stages of chromatographic purification [19]. Initial lipid class fractionation using normal-phase LC was followed by interrogation of the phosphatidylcholine fraction on a C₁₈ reversed-phase column with online detection by MS. These analyses revealed well-resolved chromatographic peaks corresponding to the phospholipid isomers PC 16:0_18:1(*n*-9) and PC 16:0_18:1(*n*-13) that differ only in the position of the double bond in the monounsaturated 18:1 acyl chain. The electrospray ionization (ESI) mass spectra of such isomeric lipids are identical, while tandem mass spectra obtained on most commercial instruments are also similar [20]. In order to identify the double bond positions within each isomer, Brouwers *et al.* collected fractions for each isomer and subjected them to further, offline interrogation including the use of high-energy collision-induced dissociation [19]. This study serves to highlight that chromatographic separation alone is insufficient for structure elucidation. Ideally, reliable methods for the identification of double bond location in phospholipids that can be achieved in real time with chromatographic separation of isomeric phospholipids should be developed.

Curtis and co-workers have implemented an ingenious approach to on-line lipid isomer analysis by using a gas-permeable membrane to allow ozone to interact with unsaturated lipids in the LC eluent prior to MS analysis [21,22]. Ozone reacts with carbon-carbon double bonds to bring about oxidative cleavage and mass spectral analysis of the products enables the assignment of double bond position(s). Undertaking this same chemistry inside the mass spectrometer itself may have advantages in that the diagnostic products of ozonolysis can be attributed to a single, mass-selected population of ionized lipids. We have previously demonstrated that mass-selected ionized lipids react with ozone in the gas phase in a process referred to as ozone-induced dissociation (OzID) [23-25]. The oxidative cleavage of carbon-carbon double bonds in the mass spectrometer gives rise to neutral losses that are characteristic of double bond position and can be used for structural assignment. OzID has previously been deployed in combination with direct infusion electrospray ionization protocols (*i.e.*, “shotgun lipidomics”) to characterise lipids in complex extracts derived from a range of sources including human ocular lens tissue, very-low density lipoprotein and insect pheromones [26-28]. The direct infusion approach – widely used in lipidomic workflows [29] – has the advantage of providing relatively long acquisition times for OzID but has the disadvantage, in some instances, of greater spectral complexity. It would be desirable therefore to be able to undertake OzID analysis within both direct infusion and LC-MS workflows with the later requiring significant re-optimisation of experimental protocols. Here, for the first time, we demonstrate the successful combination of OzID with reversed-phase LC for the separation and on-line identification

of phospholipid double bond isomers. The method was optimized on simple binary mixtures of synthetic phospholipid isomers and then challenged by more complex biological extracts.

Materials and Methods

Materials

All solvents used, including water, were Optima LCMS grade and purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia). Formic acid (98% pure); sodium citrate ($\geq 99\%$ pure); and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate and sodium acetate (both analytical grade) were purchased from Ajax Chemicals (Auburn, NSW, Australia). Industrial-grade compressed oxygen was obtained from BOC (Cringila, NSW, Australia).

Synthetic phospholipid standards PC 18:1(*n*-12)/18:1(*n*-12) [1,2-di-(6*Z*-petroselinoyl)-*sn*-glycero-3-phosphocholine] and PC 18:1(*n*-9)/18:1(*n*-9) [1,2-di-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphocholine], were acquired from Avanti Polar Lipids (Alabaster, Alabama, USA). L- α -Phosphatidylcholine from chicken egg yolk was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sheep brain was purchased frozen from Kieraville Butchery (Kieraville, NSW, Australia).

Sample Preparation

For direct infusion experiments used to optimize the mass spectrometer the lipid standard, PC 16:0/18:1(*n*-9), was dissolved to a final concentration of 30 μM in water/acetonitrile/2-propanol (50/25/25, v/v) with 1 mM sodium acetate. The working solution of egg yolk extract was prepared in the same manner with an estimated total PC concentration of 175 μM .

For chromatographic analyses, standards PC 18:1(*n*-9)/18:1(*n*-9) and PC 18:1(*n*-12)/18:1(*n*-12) were mixed at a 1:1 ratio and diluted to a final concentration of 40 μM each in water/acetonitrile/2-propanol (50/25/25, v/v). Egg yolk PC extract was prepared in the same way as for direct infusion experiments but without the addition of sodium acetate. The sheep brain was dissected to obtain one sample each of white matter and grey matter from the temporal lobe of the cerebrum. After dissection, 20-25 mg of each sample was processed using a bead homogenizer (FastPrep-24, MP Biomedical, Seven Hills, NSW, Australia) containing glass beads with a 1 mm diameter. Total lipids were then extracted using methanol/chloroform according to a modified Folch extraction method [30,31]. The lipid extracts from white and gray matter were pooled and the combined extract was washed 3 times with aqueous ammonium acetate (65 mM), dried under a stream of nitrogen and finally reconstituted with 750 μL of 2-propanol/acetonitrile (50/50, v/v). All prepared stock samples were stored at -80°C until analysis.

Chromatography

All chromatography was performed using a Waters Acquity UPLC system (Waters, Milford MA, USA). For analysis of PC standards, mobile phase A was composed of water, formic acid (0.05%, v/v) with sodium citrate (50 μ M) and mobile phase B was composed of acetonitrile/2-propanol (64/34, v/v), formic acid (0.05%, v/v) and sodium citrate (50 μ M). For the analysis of egg yolk and sheep brain extracts, mobile phase A was composed of water with sodium acetate (100 μ M) and mobile phase B was composed of acetonitrile/2-propanol (75/25, v/v) with sodium acetate (100 μ M).

Injections of the PC standard mixture (10 μ L); PC egg yolk extract (7.5 μ L); and sheep brain extract (7.5 μ L) were loaded onto a reversed-phase Acquity C₁₈ BEH column (1.7 μ m, 150 mm x 1 mm I.D., Waters, Milford MA, USA). For the analysis of PC standards, the column was heated to 50 °C with a constant flow rate of 100 μ L/min. A linear gradient was employed ramping mobile phase B from 40.0% to 99.9% over 60 min and then holding B at 99.9% B for 10 min before the column was re-equilibrated at 40% mobile phase B for 15 min. For the analysis of egg yolk PC extract and sheep brain extract, the flow rate was set to 125 μ L/min. A linear gradient was employed ramping mobile phase B from 82.0% to 88.0% over 70 min and then holding mobile phase B at 99.9% for 5 min before the column was re-equilibrated for 15 min at 82% mobile phase B. All extracts were stored at 5 °C for the duration of the LC analyses and discarded after all LC analyses were complete.

Mass Spectrometry

Mass spectrometry was undertaken on a hybrid triple quadrupole linear ion-trap mass spectrometer (QTRAP 2000, AB SCIEX, Concord, ON, Canada) that has been modified for OzID experiments as previously described [24]. Minor adjustments were made to the online ozone delivery system and a schematic showing the configuration used in the present experiments is provided in Electronic Supplementary Materials (see Figure S1). Ozone was generated from oxygen at *ca.* 170 g m⁻³ (normal) using an ozone generator (Titan 100 generator, Absolute Ozone, Edmonton Canada) at a constant flow of 0.2 L/min and was introduced via a variable leak valve (VSE Vacuum Technology, Lustenau, Austria).

All mass spectrometry was conducted in positive ion mode. For direct infusion electrospray ionization experiments, synthetic lipid standards dissolved in a solvent system comprised of water, acetonitrile and isopropanol were infused at 8 μ L/min with a declustering potential of 110 V and the source temperature was set to 70 °C. For on-line LC-MS protocols declustering potentials of 60 and 90 V and source temperatures of 200 and 350 °C were used for examining lipid standards and extracts, respectively (see LC methods above). Instrument parameters for OzID were controlled using a modified method file as previously described [24]. Typical conditions were: unit mass

resolution on the first quadrupole (see Q1 in Electronic Supplementary Materials Figure S1); pre-/post-reaction activation energies of 5/30 eV (except as noted); multi-channel acquisition was turned on; fill time for ions entering the collision cell (see q2 in Figure S1) was 50-200 ms with pre-/post-cell lenses (see lenses marked IQ2 and IQ3 in Figure S1) set to 25/100 V; and reaction time was 2000-4000 ms. For LC-MS analyses of biological samples, the alternating full scan (Q3 ion-trap scan) and OzID scans were undertaken throughout the experiment.

Nomenclature

Lipid nomenclature used here is guided by the recommendations of the Lipid MAPS consortium and the recent suggestions of Liebisch *et al.* for mass spectrometry derived data [32,33]. It is instructive for OzID analysis however, to annotate double bond position using the traditional nomenclature “*n*-*x*” where “*n*” refers to the number of carbon atoms in the chain and subtracting “*x*” provides the location of the double bond relative to the methyl terminus [34]. For example, 18:1(9Z) becomes 18:1(*n*-9) and 18:1(11Z) becomes 18:1(*n*-7). Note that this nomenclature does not define the stereochemical configuration of the carbon-carbon double bond (*i.e.*, *cis* or *trans*): a structural feature not assigned by OzID without reference to standards.

Results and Discussion

Optimization of OzID Conditions

To optimize the mass spectrometry conditions for OzID, a solution containing a commercial egg yolk extract was infused directly into the ESI source operating in positive ion mode. Source and solution conditions were optimized for the production of, $[M+Na]^+$, sodium adduct ions of lipids as these have been shown previously to react more rapidly with ozone than protonated analogues [35]. Under these conditions, abundant ions at m/z 782 corresponding to the $[M+Na]^+$ ion of PC 34:1 were observed and subsequently mass-selected using the first quadrupole. The ions were transmitted to the collision cell where they were trapped in the presence of ozone for 2000 ms before being transferred to the linear ion trap for mass analysis. Representative OzID spectra obtained from the m/z 782 ion population are shown in Figure 1. Figure 1(a) was obtained using translational energies of 37 eV for ions entering the collision cell and the application of a post-reaction energy of 15 eV. Peaks corresponding to primary OzID ions are observed at m/z 672 and 688 (marked with a filled square, ■) corresponding to neutral losses of 110 and 94 Da, respectively, that are diagnostic for a carbon-carbon double bond at an $n-9$ position (see Electronic Supplementary Materials Table S1) [20]. In addition, a number of abundant ions arising from collision-induced dissociation (CID) are observed at m/z 723, 599 and 577 that are characteristic of the $[M+Na]^+$ ions of phosphatidylcholines [36]. Taken together, the combination of OzID and CID features in the spectrum allows the assignment of PC 16:0_18:1($n-9$) as the major lipid at this mass-to-charge ratio.

[Figure 1]

Interestingly, many of the CID ions in Figure 1(a) also have associated OzID peaks. For example, under these conditions the CID product ion at m/z 723 undergoes OzID to yield product ions at m/z 613 and 629 with the same characteristic neutral losses of 110 and 94 Da, respectively. In addition, very abundant ions such as the base peak at m/z 379 can be attributed to the recently described sequential CID/OzID processes (see also other ions marked by an open square (□) in Figure 1a) [37]. This complex array of CID and OzID ions can be instructive of the structure of the lipids in question, including assigning the position of acyl chains on the glycerol backbone [25,38,39,37,40,41]. However, in the present study where our aim was to combine LC with OzID, we focused on concentrating the signal in product ions carrying information on the carbon-carbon double bond position. To achieve this the translational energy of ions entering the collision cell was minimised to 5 eV (to decrease primary CID processes) while the energy of ions exiting the collision cell was raised to 30 eV to convert most OzID ions through to a single set of abundant products [24].

This approach is illustrated in Figure 1(b) where the base peak is m/z 489 which, along with the associated m/z 505 ion, can be assigned as OzID ions of the $[M+Na]^+$ precursor ion that have subsequently undergone CID with loss of the phosphocholine headgroup (see suggested mechanism in Scheme 1). The improvement in the abundance in the diagnostic ions is apparent by considering the m/z 498 in Figure 1(b) is almost 2-fold greater than m/z 672 in Figure 1(a). More importantly however, the signal-to-noise in these desired mass channels is enhanced with low abundant signals at m/z 517 and 531 in Figure 1(b) now discernible above the noise. These OzID ions are indicative of the presence of a small amount of an $n-7$ isomer, most likely PC 16:0_18:1($n-7$) and a contribution from the $[M+2]$ isotopologue of PC 16:0_18:2($n-6,n-9$). The instrument conditions used to obtain the OzID spectrum in Figure 1(b) were also used in the online LC-OzID experiments described below.

While it is beyond the scope of the current study, optimization of experimental conditions for other classes of glycerophospholipids (or indeed other lipid classes) could be readily undertaken using the same procedure as that documented here for phosphatidylcholines.

[Scheme 1]

LC-MS of synthetic phospholipid isomers

Two synthetic phosphatidylcholines, PC 18:1($n-9$)/18:1($n-9$) and PC 18:1($n-12$)/18:1($n-12$), were combined as a 1:1 mixture and subjected to HPLC using a reversed-phase C_{18} column (see Methods section). Positive ion ESI of the eluent yielded abundant m/z 808 ions (*i.e.*, the $[M+Na]^+$ forms of the two isomeric lipids) that were analysed by OzID (Figure 2).

[Figure 2]

The total ion chromatogram (TIC) constructed from the abundance of all ions detected in OzID scans of m/z 808 is shown in Figure 2(a) and reveals two distinct chromatographic features of near equal abundance. Prior observation of the behaviour of unsaturated phosphatidylcholines on reversed phase columns suggests that the peaks eluting at 49.0 and 50.4 min correspond to the isomers PC 18:1($n-9$)/18:1($n-9$) and PC 18:1($n-12$)/18:1($n-12$), respectively [17,19,18]. Integration of OzID scans obtained across each of the chromatographic peaks gave the OzID mass spectra shown in Figures 2(b) and (c). The base peak in both mass spectra is at m/z 625, which corresponds to the neutral loss of 183 Da (*i.e.*, loss of the choline headgroup) from the precursor ion at m/z 808 [36]. Distinctive product ion pairs at m/z 515, 531 (marked as ■ in Figure 2b) and m/z 473, 489 (marked as ◆ in Figure 2c) are observed in the OzID spectra of the early-eluting and late-eluting

chromatographic features, respectively. These can be assigned to OzID transitions of 110, 94 Da and 152, 136 Da from the base peak at m/z 625 that are characteristic of n -9 and n -12 double bond positions, respectively [25]. The bulk of these diagnostic ions are formed *via* sequential OzID then CID activations illustrated in Scheme 1 and enable unambiguous confirmation of the putative assignments of the chromatographic peaks to the n -9 (49.0 min) and n -12 (50.4 min) isomers. The uniqueness of the OzID product ions to each isomer is illustrated in Figure 2(d) where the extracted ion chromatograms (XICs) of m/z 515 (shown as blue trace) and the m/z 473 (shown as a red trace) are shown to be fully resolved. It is interesting to note that, despite the fact that the isomers were prepared as a 1:1 mixture and show similar peak areas in the total ion chromatograms in Figure 2(a), the peak area of the m/z 515 selected ion chromatogram is approximately double that of the m/z 473 peak. This phenomenon arises from the different rates of reaction of the two ionized lipid isomers with ozone in the gas phase. The general trend of faster ozonolysis reactions with double bonds closer to the methyl terminus of lipid acyl chains has previously been noted [24]. This is consistent with the current observation where double bonds at the n -9 position react faster than those at the n -12 position. Importantly, this result suggests that while ion chromatograms generated from OzID transitions can provide an excellent means to identify and resolve overlapping double bond isomers, relative quantification using these data alone cannot be achieved without calibration of the relative ozonolysis reaction efficiencies of the isomers.

LC-MS of complex lipid extracts

Upon optimizing the LC-OzID workflow, this protocol was used to examine lipid extracts from chicken egg yolk and sheep brain. The phosphatidylcholine composition of such extracts is known to be rich in PC 16:0_18:1, although the position(s) of unsaturation have not been fully elucidated [42,43].

While the chicken egg yolk extract is a relatively simple lipid mixture (*i.e.*, a phosphatidylcholine fraction), it is substantially more complex than the standard mixtures described above. As a result of the increased complexity the chromatographic conditions were adjusted as described above (see Methods). **Total ion chromatograms showing the elution of all components of the mixture detectable under the experimental conditions are provided as Electronic Supplementary Materials (see Figure S2)** and also provide evidence for the reproducibility of the conditions across two injections. The chromatographic and mass spectral data obtained from OzID of m/z 782, corresponding to the $[M+Na]^+$ ion formed from PC 34:1, is shown in Figure 3. In the chromatographic region of interest in Figure 3(a) the early-eluting components at retention times of 21.3 and 22.4 min are assigned to $[M+2]$ isotope contributions from PC 34:2. This assignment was confirmed by

examining the full-scan mass spectra obtained at these elution times that were dominated by ions at m/z 780 (data not shown). The major peak in Figure 3(a) is centred at 31.3 min and features a discernible shoulder centred at approximately 30.4 min. The OzID spectrum of m/z 782, obtained from averaging the scans across the main chromatographic peak, is shown in Figure 3(c). This spectrum has a single major set of OzID product ions at m/z 489, 505 (marked with ■) corresponding to transitions of 110, 94 Da from the m/z 599 base peak. The formation of these ions can be rationalised according the sequence of OzID and CID events depicted in Scheme 1 and confirms the presence of an n -9 double bond in the eluting phosphatidylcholines. In contrast, the OzID spectrum obtained from the smaller chromatographic feature centred at 30.4 min is dominated by the product ion pair of m/z 517, 533 (marked with * in Figure 3b) corresponding to transitions of 82, 66 Da from the m/z 599 base peak. These neutral losses have previously been reported to arise from oxidative cleavage of an n -7 double bond during OzID (see Electronic Supplementary Materials Table S1 for a summary of characteristic OzID transitions) [44,24,25]. The n -9 OzID product ion at m/z 489 is also observed, albeit at low abundance, in the spectrum shown in Figure 3(b) and arises from the incomplete chromatographic separation of two isomeric lipids that differ in the position of unsaturation. Support for this is provided by the overlay of ion chromatograms extracted from the diagnostic OzID ions at m/z 489 (n -9 marker ion, red trace) and m/z 517 (n -7 marker ion, blue trace) shown in Figure 3(d).

[Figure 3]

While LC-MS/MS data has previously been used to assign the sum composition PC 34:1 in chicken egg yolk to the fatty acyl composition PC 16:0_18:1, those results provided no indication of the presence of double bond positional isomers [42]. Conversely, the LC-OzID data presented here demonstrate that the PC 34:1 lipid population in this extract is a mixture of the double bond positional isomers PC 16:0_18:1(n -9) and PC 16:0_18:1(n -7). While, the chromatographic resolution of these isomers is incomplete, we estimate that PC 16:0_18:1(n -9) is some 4-5 times more abundant than PC 16:0_18:1(n -7) (Figure 3a).

The incomplete chromatographic resolution of the PC 16:0_18:1 isomers in egg yolk extract stands in contrast to the complete separation of the binary mixture of synthetic double bond isomers of PC 18:1_18:1 (Figure 2a). The difference in chromatographic performance arises in part because of the greater complexity of the biological extract and the greater structural differences between each isomeric pair (*i.e.*, two double bonds versus one). This also suggests that separation of double bond positional isomers on reversed-phase columns becomes easier as the spacing between

the sites of unsaturation increases and if double bonds are closer to the carboxylate moiety (*cf.* separation of *n*-9 and *n*-12 isomers in Figure 2a with separation of *n*-7 and *n*-9 isomers in Figure 3a) [19]. It is also interesting to note that the relative peak areas in the extracted ion chromatograms in Figure 3(d) are actually representative of the total ion chromatograms shown in Figure 3(a). This suggests that the ozonolysis rates of the sodiated PC 16:0_18:1(*n*-9) and PC 16:0_18:1(*n*-7) isomers are similar. This is in contrast to the pattern observed for the PC 18:1(*n*-9)/18:1(*n*-9) and PC 18:1(*n*-12)/18:1(*n*-12) (Figure 2) where a clear bias was observed. This finding suggests that for the PC 16:0_18:1 isomers, extracted OzID traces could be used for approximating relative abundance where chromatographic resolution alone is insufficient (see below).

Lipid extracts from sheep brain tissue are substantially more complex mixture than phosphatidylcholine fractions from egg yolk. Previous investigations in our laboratory have shown phosphatidylcholines of composition PC 34:1 are abundant in this tissue and traditional tandem mass spectrometry has been used to assign the acyl chain composition to PC 16:0_18:1 [43]. LC-MS analysis of sheep brain extract was undertaken using the methods described above and the results are summarised in Figure 4.

[Figure 4]

The chromatogram extracted from the total abundance of ions detected from OzID scans of *m/z* 782 (Figure 4a) shows a low abundance feature centred at a retention time of *ca.* 22.3 min. This retention time is similar to that observed previously for the [M+2] isotope contributions from PC 34:2 (*cf.* Figure 3a) and this was confirmed by observation of the *m/z* 780 base-peak in the full scan mass spectra obtained at this retention time (data not shown). The chromatogram in Figure 4(a) is dominated by a large broad peak stretching from 30-33 min and, by analogy to the results obtained from egg yolk, this feature is assigned to isomers of PC 16:0_18:1. The absence of clear chromatographic resolution however, makes the assignment of this peak more challenging. Integration of the OzID scans obtained from the early (30.2-30.8 min) and late (32.0 – 32.6 min) eluting portions of the peak yielded the OzID mass spectra shown in Figures 4(b) and (c), respectively. These spectra are clearly distinct and reveal the characteristic OzID ion pairs of *m/z* 517, 533 (corresponding to an *n*-7 double bond) and *m/z* 489, 505 (diagnostic for an *n*-9 double bond, see Table S1). These data suggest that the chromatographic feature is a composite of the two double bond-positional isomers namely, PC 16:0_18:1(*n*-7) and PC 16:0_18:1(*n*-9). Extracted ion chromatograms of two of these characteristic transitions, *m/z* 517 (shown as a blue trace, Figure 4d) and *m/z* 498 (shown as a red trace, Figure 4d), reveal the different chromatographic behaviour of

the two isomers. The *n*-7 isomer (30.9 min) elutes earlier than the *n*-9 isomer (31.8 min), which is analogous to the trend observed for the egg yolk extract (Figure 3a). Overall however, the isomer resolution for the sheep brain extract is lower than that achieved for egg yolk. The difference in chromatographic performance can be attributed to (i) the greater overall complexity of the sheep brain extract, which has significant contributions from all major classes of phospholipid [43], but also (ii) the apparently greater contribution of the *n*-7 isomer in sheep brain. As noted above, for the comparison of *n*-7 and *n*-9 isomers of PC 16:0_18:1 might be expected to give similar detection efficiencies in their respective OzID transitions. As such, the relative proportions can be estimated from Figure 4(d), suggesting that the ostensibly less common *n*-7 isomer might be present at up to 50% of the PC 16:0_18:1(*n*-9) species.

To assess the efficacy of this method for analyzing more complex lipids, the egg yolk extract was also interrogated for isomers of polyunsaturated phosphatidylcholines of sum composition PC 34:2. LC-MS results from this analysis are summarised in Figure 5. The total abundance of all ions observed in the OzID scans for *m/z* 808, corresponding to the $[M+Na]^+$ ion of PC 34:2, are plotted in Figure 5(a) and show three distinct features. The least abundant chromatographic peak, centred at 22.5 min, is assigned to the $[M+2]$ isotope contribution of PC 34:3 (a lipid previously identified in egg yolk [42]). The contribution of these phosphatidylcholines was confirmed by the presence of abundant *m/z* 806 ions in the full mass spectra obtained at this retention time (data not shown). The two major chromatographic peaks in Figure 5(a) are baseline resolved and are centred at 31.4 and 32.8 min. OzID spectra obtained from averaging scans across these two peaks are shown in Figures 5(b) and (c), respectively. The OzID spectrum shown in Figure 5(b) is nearly identical to that obtained from the synthetic standard, PC 18:1(*n*-9)/18:1(*n*-9) (see Figure 2b). Indeed, even the relative abundance ratio of the diagnostic OzID ions at *m/z* 515 and 531 is identical (*ca.* 3:1), suggesting that not only are both double bonds at the *n*-9 position but they also carry *cis*-stereochemistry [24]. The OzID spectrum shown in Figure 5(c) reveals two sets of OzID ions at *m/z* 517, 533 (marked with ■) and *m/z* 557, 573 (marked with ▲), representing neutral losses from the *m/z* 627 base peak that are consistent with the *n*-9 and *n*-6 double bond positions in the unsaturated acyl chain of PC 16:0_18:2(*n*-6,*n*-9) [25]. Both the PC 18:1_18:1 and PC 16:0_18:2 acyl chain compositions were previously assigned based on conventional tandem mass spectral analysis on egg yolk extract [42]. The LC-OzID results presented here enable the separation of these isomeric forms with unambiguous assignment of double bond positions in each case (*e.g.*, see XICs in Figure 5d).

[Figure 5]

Conclusions

The results presented here demonstrate that it is possible to obtain high quality OzID mass spectra on a timescale that is compatible with conventional LC. While OzID scans remain intrinsically slow compared to CID experiments, we have shown here that it is possible to obtain up to 20 OzID scans, interleaved with other scan functions, during the elution of a chromatographic peak (*ca.* 1 min). When combined with other mass spectral and chromatographic information, this hybrid workflow enables the structural elucidation of complex lipids and, importantly, the identification of distinct isomeric forms of lipids differing only in the position(s) of carbon-carbon double bonds. In complex extracts where chromatographic separation of such isomers is poor, OzID enables the LC profiles of each isomer to be deconvoluted and, in some instances, the relative proportions of each isomer can be approximated. This hyphenated LC-OzID approach has some advantages over infusion in simplifying OzID spectra through minimizing isomer overlap and removing contributions of [M+2] isotopes. While the methods used here targeted specific phosphatidylcholines of interest, examples provided as Electronic Supplementary Materials (Figures S3 and S4) demonstrate that (i) the method can be applied to lower abundance, polyunsaturated lipids and (ii) LC-OzID spectra with good signal-to-noise can be achieved on even shorter timescales should future applications require faster chromatography or the interrogation of more molecular species. OzID has previously been shown to work effectively on a wide variety of lipid structures in infusion ESI experiments [23] so the application of LC-OzID to the examination of other classes (*e.g.*, other glycerophospholipids, sphingolipids, triacylglycerols) represents a ready extension of the workflows described herein. Future workflows for LC-OzID, will take advantage of information dependent approaches where OzID scans are triggered based on precursor ion detection in a preceding full scan analysis. The ability to conduct the ozonolysis event using data-dependent selection criteria (*e.g.*, inclusion lists, exclusion lists, tandem mass spectral criteria such as neutral losses, *etc.*) will ensure that the majority of unsaturated lipids are detected and the position(s) of unsaturation within their acyl chains are established.

Under the conventional reversed-phase chromatographic conditions used here, the closer the double bond of the acyl chain to the carboxylate group, the more strongly it was retained on the column (*cf.* *n*-9 elutes before *n*-12, Figure 2a). This observation is consistent with previous reports of separation of double bond positional isomers and evidence from new column technologies suggests further improvements in isomer separation can be achieved [17,19,18]. Full structure elucidation of complex lipids however, also requires separation and/or mass spectral differentiation of isomers that differ in (i) the position of acyl chains on the glycerol backbone (*i.e.*, *sn*-positional isomers) and the stereochemistry about the double bond(s) (*i.e.*, *cis* and *trans*). Some preliminary reports have

suggested that separation of such isomers is possible in selected circumstances but it is by no means routine [45,46]. Perhaps the addition of complementary mechanisms of separation, such as ion-mobility spectrometry with LC and mass spectrometry may provide a means to achieve full structure assignments for lipids in complex biological extracts [47,39,48].

Although OzID and other technologies reveal the presence of multiple isomeric forms of phospholipids within biological samples, very little is currently understood about the biological role and/or evolutionary reason for these isomers [20,25]. Future application of LC-OzID to extracts from different biological sources will aid in the identification of the presence of isomeric lipids and may assist in revealing changes in isomer populations that will provide essential clues as to the different role(s) of these isomers *in vivo*.

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Electronic Supplementary Materials:

Additional information on methods and chromatographic data are provided along with a look-up table for characteristic OzID neutral losses.

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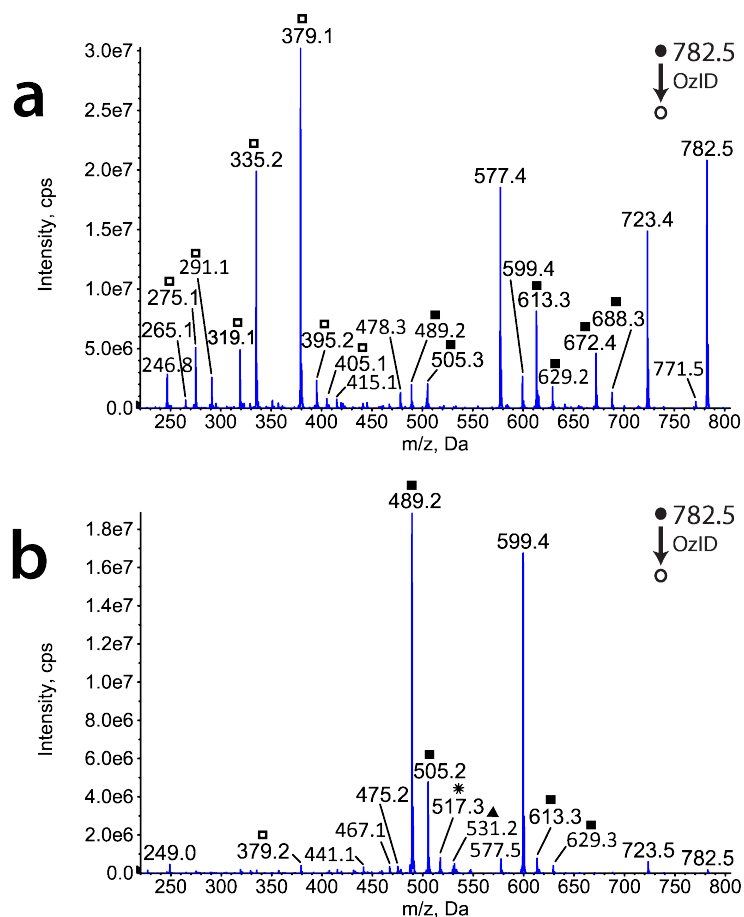


Figure 1. OzID spectra obtained from m/z 782.5 (predominantly the $[M+Na]^+$ ions of PC 34:1) produced upon positive-ion electrospray ionization of a solution containing a commercial egg yolk extract. (a) The OzID spectrum obtained using pre- and post-reaction energies of 37 eV and 15 eV, respectively. (b) The OzID spectrum obtained using pre- and post-reaction energies of 5 eV and 30 eV, respectively. OzID product ions indicative of an $n-9$ (■), $n-7$ (*) and $n-6$ (▲) are marked with different symbols while ions arising from the recently described CID/OzID processes are also marked (□) [37].

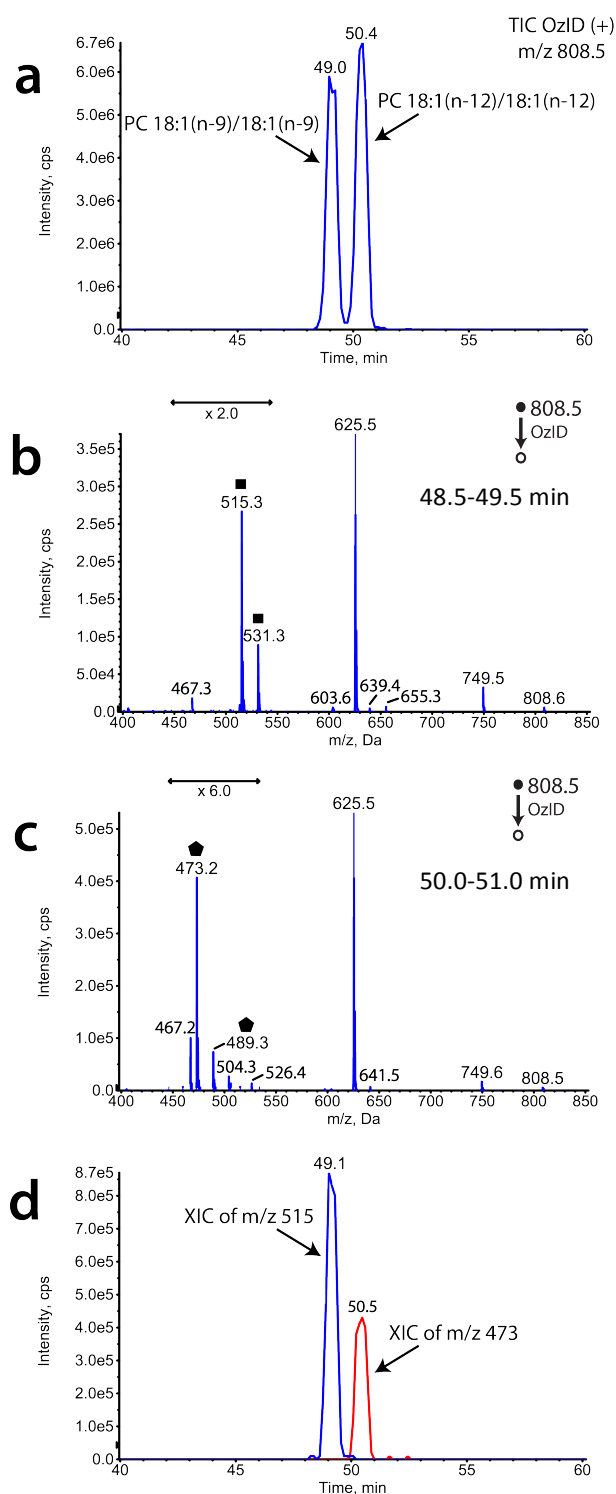


Figure 2. Results of LC-MS analysis of a 1:1 mixture of the synthetic phospholipid isomers, PC 18:1(*n*-9)/18:1(*n*-9) and PC 18:1(*n*-12)/18:1(*n*-12). (a) The region of interest of the chromatogram constructed from total abundance of ions detected in OzID scans of mass-selected *m/z* 808.5. The OzID mass spectra obtained by integrating all scans between (b) 48.5-49.5 min and (c) 50.0-51.0 min. (d) Extracted ion chromatograms (XICs) for OzID product ions at *m/z* 515 (blue trace) and *m/z* 473 (red trace). OzID product ions indicative of *n*-9 (■) and *n*-12 (◆) double bond positions are marked with the symbols shown.

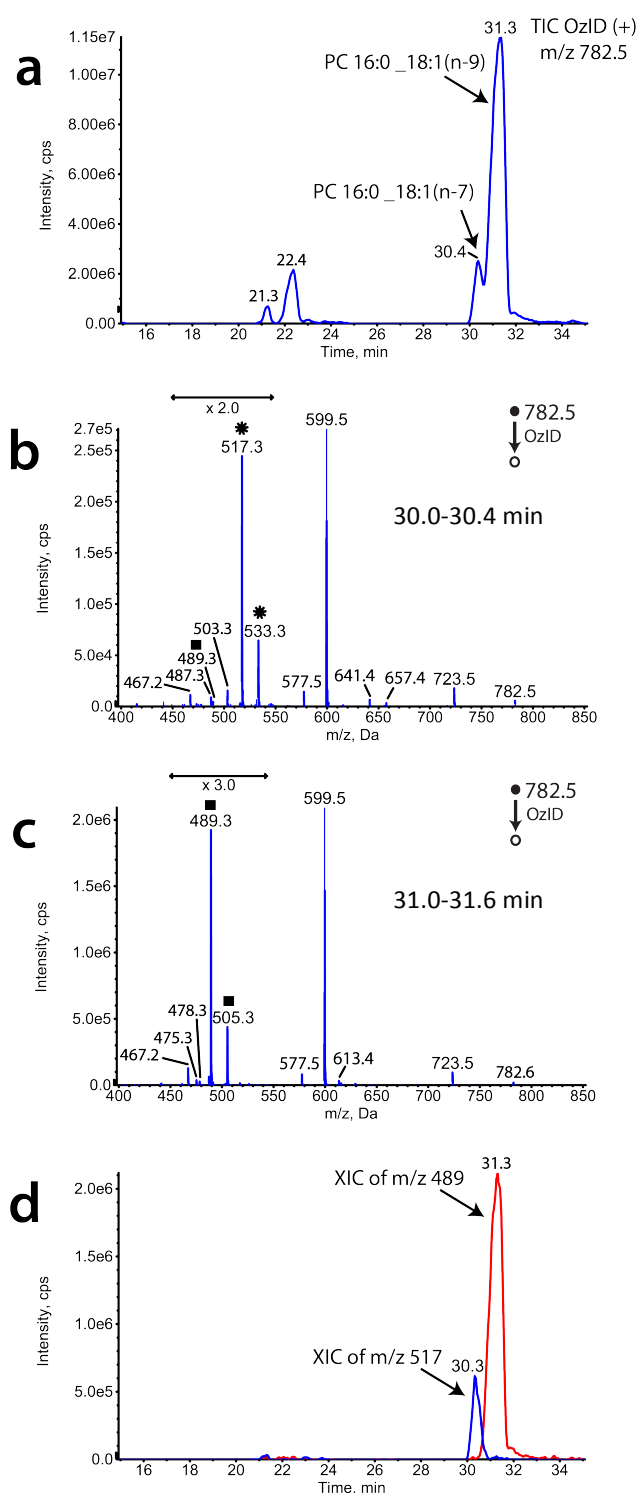


Figure 3. Results of LC-MS analysis of a purified phosphatidylcholine extract from egg yolk. (a) The region of interest of the chromatogram constructed from total abundance of ions detected in OzID scans of mass-selected m/z 782.5. The OzID mass spectra obtained by integrating all scans between (b) 30.0-30.4 min and (c) 31.0-31.6 min. (d) Extracted ion chromatograms (XICs) for OzID product ions at m/z 517 (blue trace) and m/z 489 (red trace). OzID product ions indicative of n -7 (✱) and n -9 (■) double bond positions are marked with the symbols shown.

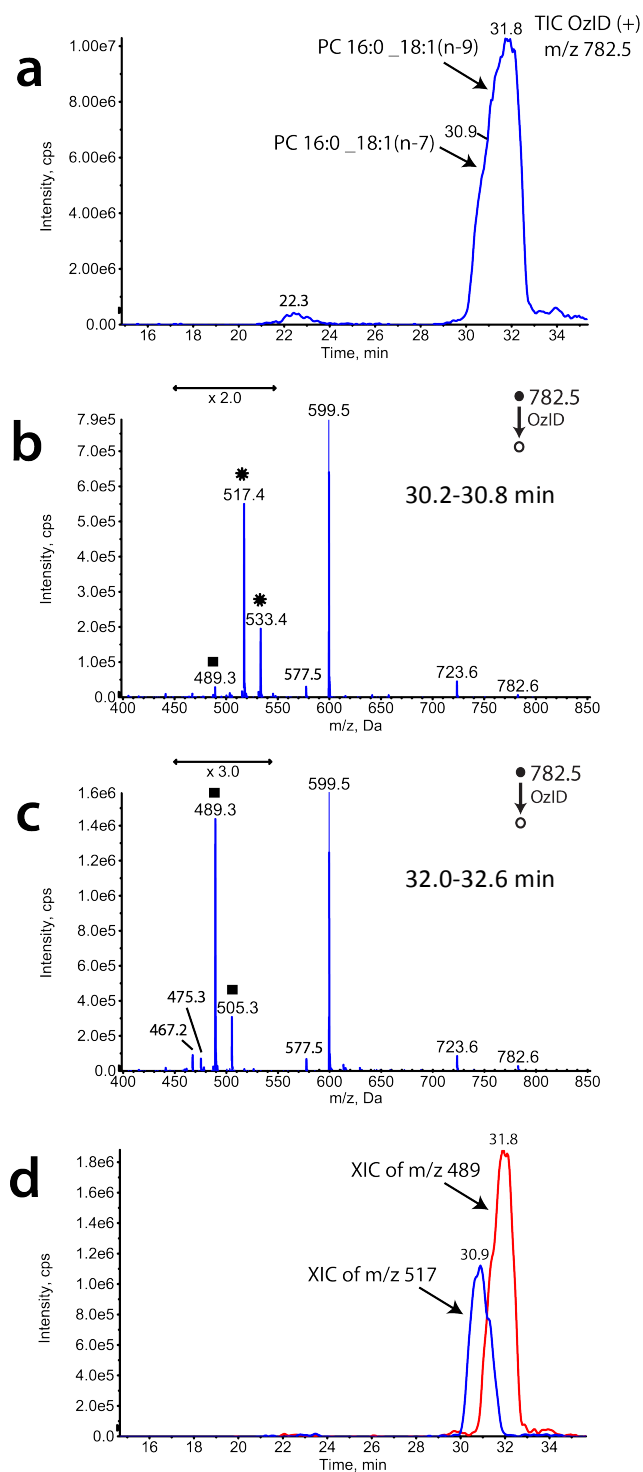


Figure 4. Results of LC-MS analysis of a lipid extract from dissected sheep brain tissue. (a) The region of interest of the chromatogram constructed from total abundance of ions detected in OzID scans of mass-selected m/z 782.5. The OzID mass spectra obtained by integrating all scans between (b) 30.2-30.8 min and (c) 32.0-32.6 min. (d) Extracted ion chromatograms (XICs) for OzID product ions at m/z 517 (blue trace) and m/z 489 (red trace). OzID product ions indicative of n -7 (*) and n -9 (■) double bond positions are marked with the symbols shown.

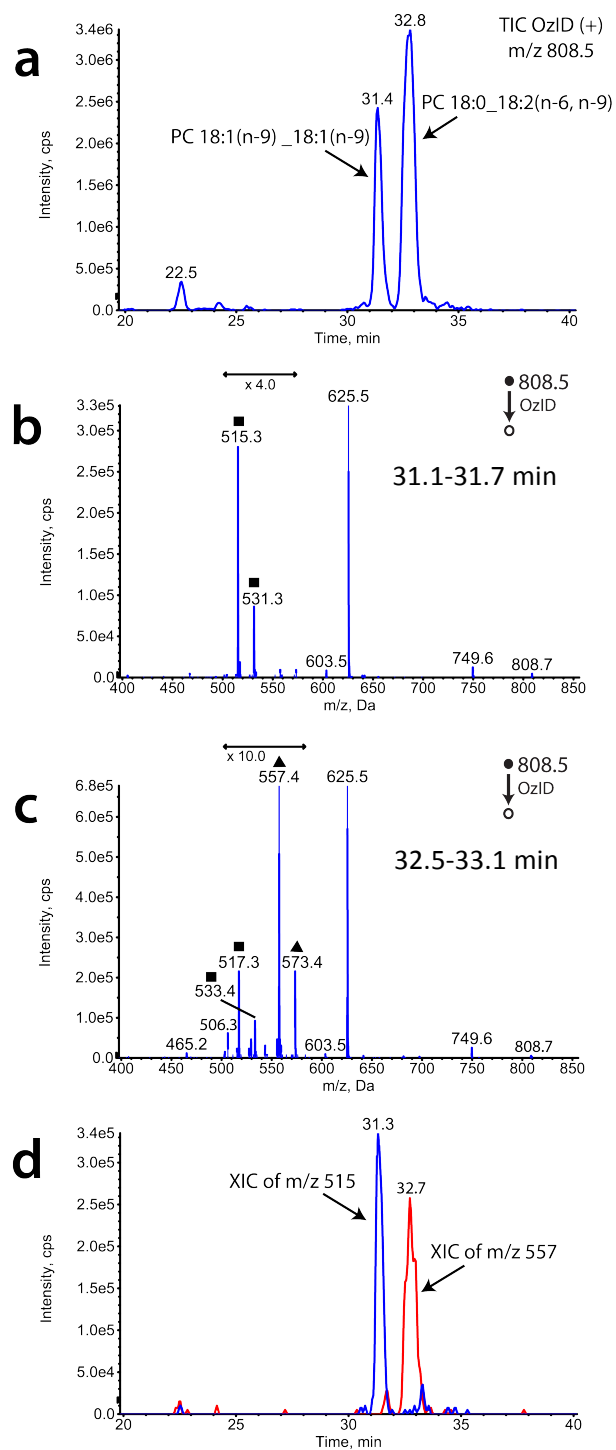


Figure 5. Results of LC-MS analysis of a purified phosphatidylcholine extract from egg yolk. (a) The region of interest of the chromatogram constructed from total abundance of ions detected in OzID scans of mass-selected m/z 808.5. The OzID mass spectra obtained by intergrating all scans between (b) 31.1-31.7 min and (c) 32.5-33.1 min. (d) Extracted ion chromatograms for OzID product ions at m/z 515 (blue trace) and m/z 557 (red trace). OzID product ions indicative of n -6 (▲) and n -9 (■) double bond positions are marked with the symbols shown.

Scheme 1. Fragmentation of the $[M+Na]^+$ precursor ion formed from PC 16:0/18:1(*n*-9) by sequential ozone- and collision-induced dissociation processes to yield abundant product ions diagnostic of carbon-carbon double bond position in the target lipid.

